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Growth of *Chlorella vulgaris* using wastewater from Nile tilapia (*Oreochromis niloticus*) farming in a low-salinity biofloc system

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ABSTRACT. The present study aimed to analyze the growth of *Chlorella vulgaris* in the effluent of a Biofloc Technology (BFT) system used in the Nile tilapia fingerlings farming. The conditions were cultivated by 10 days at 25 ± 2°C, 90 µmol photons m^{-2} s^{-1} irradiance, under constant aeration (without addition of CO_2), and with different BFT effluent proportions (0, 50, and 100%). After 24 hours of the inoculation that marked the beginning of the experiment, the development and multiplication of the cells were verified, demonstrating that the BFT effluent used in the Nile tilapia farming was favorable for the cultivation of the microalga *Chlorella vulgaris*. As expected, the Provasoli culture medium presented the best performance (1,520 ± 75 10^4 cells mL^{-1}) in relation to the growth of the microalga. However, during the first four days of cultivation, *C. vulgaris* showed higher growth in treatments containing BFT effluent. *C. vulgaris* removed 93.6% of the nitrate contained in the BFT effluent. The results of the present study showed the potential use of *C. vulgaris* on Integrated Multi-Trophic Aquaculture with Nile tilapia fingerlings. In addition to removing nitrate and other nitrogen compounds, *C. vulgaris* biomass could be used to feed zooplankton or Nile tilapia larvae.

Keywords: aquaculture; bioremediation; microalgae; wastewater treatment.

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Introduction

Tilapia is a tropical species that has shown rapid growth and high adaptability to distinct environmental conditions. The tilapia culture in cage farming systems is the most applied method worldwide, since the fish has a good growth when fed with acceptable protein levels (Costa & Fróes, 2012). However, some studies of Nile tilapia growth in Biofloc Technology (BFT) has also shown satisfactory growth and survival rate for fish farming (e.g. Azim & Little, 2008; Ekasari et al., 2015; Pérez-Fuentes, Hernández-Vergara, Pérez-Rostro, & Fogel, 2016; Zapata-Lovera, Brito, Lima, Vinatea-Arana, & Olivera, 2017).

The aquatic organisms farming in BFT systems are one of the bets in aquaculture to combat hunger (Vinatea, 2010; FAO, 2016). However, there is a discussion about the accumulated nitrogen (N) and phosphorus (P) compoundsduring farming in these systems, which are mainly from feed residues and excreta from cultivated organisms. On average, 75% of the feed-N and 50-70% of feed-P ends up in the water (Crab, Defoirdt, Bossier, & Verstraete, 2012; Lima et al., 2019). This accumulation is due to one of the main characteristics of this system, the stagnant water system (Mishra et al., 2008; Krummenauer, Cavalli, Poersck, & Wasielesky, 2011).

The microalgae, besides having an important role in the energy transfer process along the food web, present a great potential in the bioremediation of inorganic compounds (Gouveia, Raymundo, Batista, Souza, & Empis, 2006). These compounds will serve as nutrients for the microorganisms that will develop and multiply over time (Becker & Venkataraman, 1981; Kim & Wijesekara, 2010). The biomass produced from this process can be used in several purposes. Among other applications, it is worth mentioning the feed of aquatic organisms and the high value products that result from this process (Dantas et al., 2019).

Depending on the purpose and technology used to the microalgae cultivation, the process can be expensive and unfeasible, due to the high cost of nutrients used in the production, and also the need for specialized labor (Gouveia et al., 2016). The ability to assimilate and convert N and P compounds, as well as some heavy metals, into cellular components such as lipids, proteins, carbohydrates and carotenoids, induces the use of effluents as an economic and ecological alternative in production. Furthermore, most of the time, effluents are disposed without prior treatment, which cause incalculable damage to ecosystems (Miyawaki, 2014; Suali & Satabatly, 2012).

Integrated Multi-trophic Aquaculture (IMTA) promises to contribute to the sustainability of the activity, promoting a recycling of high-value nutrients (scraps feed rich in protein) that would be wasted (Troell et al., 2009). Studies on IMTA among fish, shrimp and/or seaweed were performed (e.g. Hayashi et al., 2008; Mao, Yang, Zhou, Ye, & Fang, 2009; Abreu et al., 2009; Brito et al., 2014), however, the use of microalgae in IMTA is not yet established.

Chlorella vulgaris is a Chlorophyceae with diameter of 2-10μm (Kulkarni & Nikolov, 2018), and it is a very well-known species of algae used as human food supplement (Oliveira, Almeida, Viveiros, Santos, & Dantas, 2018; Dantas et al., 2019). The *C. vulgaris* capacity in bioremediation of wastewater is already proven in the literature (Brennan & Owende, 2010; Toyama et al., 2018; Gao et al., 2019), since this species has high potential to reduce the P and N of effluents (Ruiz et al., 2011). However, there is no record of the grow capacity of this microalga in BFT effluents.

In this sense, the use of residual water from an intensive BFT system employed in Nile tilapia (*Oreochromis niloticus*) fingerlings farming was proposed as a culture medium for the *C. vulgaris*. The objective of this study was to evaluate the growth of this microalga and to monitor the assimilation of the nitrogen compounds and phosphorus in the effluent.

Material and methods

Nile tilapia farming

In order to prepare the BFT system, the marine water (10 g L^{-1}) was previously disinfected with 10 mg L^{-1} of chlorine. After three days of aeration, the water was fertilized using urea and triple superphosphate, in the ratio of 10:1, at the concentrations of 3 and 0.3 mg L^{-1}, respectively. Then, to produce biofloc, an organic fertilization was also conducted by molasses and feed (36% crude protein). The Carbon:Nitrogen ratio was maintained at 12:1 and it was calculated according to De Schryver, Crab, Defoirdt, Boon, and Verstraete (2008).

After 40 days of fertilization, the tilapia fingerlings (1 g) were stocked in the matrix tank (50 fish m^{-3}). After 15 days of the fingerling's addition, the experimental units (50-L) were filled up to ~50% of the volume using the water of matrix tank, and the remaining volume was filled with water at salinity of 10 g L^{-1}. There was no water exchange during the experimental period, except for the addition of freshwater to compensate the evaporation losses. Light intensity was kept at 45 μmol photons m^{-2} s^{-1} irradiance using a fluorescent lamp with a 12:12h (light:darkness) photoperiod.

After the end of this experiment, the three effluents were homogenized and used in the biomass production of the microalga *Chlorella vulgaris*. The water quality, centesimal composition and the data productivity of Nile tilapia fingerlings in BFT are available in Lima et al. (2019).

Microalga cultivation

The *Chlorella vulgaris* strain was supplied by the Laboratório de Produção de Alimento Vivo (*Live Food Production Laboratory*) of the Federal Rural University of Pernambuco. *C. vulgaris* was grown in 1-L Erlenmeyer glass using 0.8-L Provasoli medium (McLachlan, 1973), which contains: $NaNO_3$ 105 mg L^{-1}, Na_2EDTA 24.9 mg L^{-1}, $Na_2Glycerophosphate$ 15.0 mg L^{-1}, H_3BO_3 3.0 mg L^{-1}, $Fe(NH_4)_2(SO_4)_2$ $6H_2O$ 10.6 mg L^{-1}, $MnCl_2$ $4H_2O$ 0.6 mg L^{-1}, $FeCl_3$ $6H_2O$ 0.15 mg L^{-1}, $ZnCl_2$ 0.075 mg L^{-1}, and $CoCl_2$ $6H_2O$ 0.0015 mg L^{-1}. The BFT effluent contained NH_4 0.55 ± 0.06 mg L^{-1}, NO_2 1.23 ± 0.03 mg L^{-1}, NO_3 91.21 ± 0.03 mg L^{-1}, and PO_4 7.66 ± 0.07 mg L^{-1}. The effluent and water used in culture medium were filtered, chlorinated with 0.02 ppm sodium hypochlorite for 1.5 hours and then dechlorinated with 0.025 ppm sodium thiosulfate solution. Thereafter, both fluids were autoclaved at 100 kPa for 20 minutes, and after cooling to 24ºC, the microalga was inoculated, in order to give an initial concentration of 10^5 cells mL^{-1}.

Experimental design

The experiment was performed in 0.5-L glass flasks with different ratios of Provasoli culture medium and BFT effluent. The conditions (in triplicate) were named E0 (control), E50 and E100 for the respective quantities of effluent: 0, 50, and 100%. The cultures were acclimatized by four days under the treatment conditions before inoculation. Flasks were illuminated under a photoperiod of 24:0 (light: dark) with a 90 µmol photons m^{-2} s^{-1} irradiance. This light level was chosen to avoid any photoinhibition. All flasks were incubated at an ambient temperature of 25 ± 2°C, and the fluids were mixed with the aid of a hose, inserted in each experimental unit, which connected to a blower (Boyu air-pump S-4000B, Raoping, China) to generate air bubbles to keep the cells in suspension, without CO_2 addition.

Growth analysis

Cells counts were performed daily, using a Neubauer chamber and optical microscope (model BA300, Olympus®, Japan) with a 400x magnification. The average daily cell density data of the three replicates were obtained by the growth curve of the species in the three conditions.

The maximum cell density (MCD), doubling time (DT) and growth rate (K) parameters were evaluated, being: MCD the maximum average value of cell density obtained between the first and tenth day of culture; and the K value obtained through Equation 1, as described by Stein (1973):

$$K = [3.322\left(T_f - T_i\right)^{-1} \times \left(Log\ N_f \times N_i\right)^{-1}] \quad (1)$$

where: 3.322 = conversion factor of logarithm base 2 to base 10, (T_f-T_i) = time interval in days, N_i = initial cell density, and N_f = final cell density. For DT, which represents the time spent for division of a cell, Equation 2 was used:

$$DT = \frac{1}{K} \quad (2)$$

Water quality and nutrient removal

Water temperature, pH, salinity and dissolved oxygen (DO) were evaluated with a multiparameter (model YSI 100, Yellow Springs, USA) on the first and last day of culture. The bioremediation efficiency was calculated by the difference between NH_4, NO_2, NO_3, and PO_4 concentrations before and after microalga cultivation. Quantitative analyses of nitrogen sources, after remediation, were performed by UV-Vis spectrophotometer (model 6305, Jenway, England) according to Hansen and Koroleff (2007). Soluble phosphate analyses were performed following the ascorbic acid method (AWWA, 1992).

Statistical analysis

Data are presented as mean ± standard deviation. The normality (Shapiro-Wilk) and homogeneity (Cochran) tests were applied, and as the data were normal and homogeneous, the one-way Analysis of Variance (ANOVA) was also applied, followed by the Tukey's test for comparison of means (Zar, 2013). For all the analyses mentioned above, we considered a significance level of 5%. Data analyses were performed by R Studio software (R Studio, Inc., Boston).

Results and discussion

Growth

Cell density data over the 10 days of culture are shown in Figure 1. After 24 hours of the inoculation that marked the beginning of the experiment, the development and multiplication of the cells were verified. The data demonstrates that the effluent of the BFT system used in the Nile tilapia farming was favorable for the cultivation of the microalga *Chlorella vulgaris*. On the third and fourth day of culture, E100 treatment had the highest cell density when compared to control and E50 ($p = 0.003$). Among the fifth and eighth day of culture, there was no significant difference among the control and treatments. However, on the ninth and tenth day, the control differed significantly ($p < 0.001$) from the two other treatments, with the best growth performance.

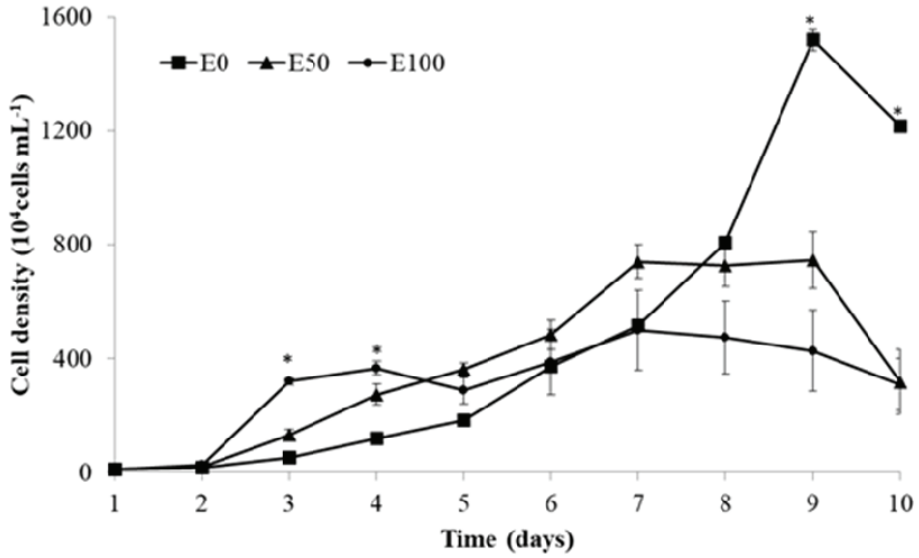

Figure 1. Cell density of *Chlorella vulgaris* over the ten days of culture. (*) show significant differences ($p < 0.05$) among the growth conditions by one-way ANOVA followed by Tukey's test.

Growth parameters data are shown in Table 1. The MCD differed significantly ($p < 0.01$) in two groups. The first one is formed by condition E0 and obtained the best performance (1,520 ± 75 10^4 cells mL^{-1}). The conditions E50 and E100 did not present significant differences between them. Regarding the parameter K, there was no significant difference ($p = 0.05$) between the treatments, a fact that is repeated for the DT parameter ($p = 0.11$).

Table 1. Mean values (± standard deviation) of maximum cell density (MCD), growth rate (K) and doubling time (DT) for *Chlorella vulgaris*.

Parameter	Treatment		
	E0	E50	E100
MCD (10^4 cells mL^{-1})	1,520 ± 75^a	971 ± 302.38^b	630 ± 236.4^b
K (div day^{-1})	0.77 ± 0.003^a	0.63 ± 0.004^a	0.53 ± 0.100^a
DT (days)	1.30 ± 0.003^a	1.66 ± 0.204^a	1.93 ± 0.374^a

Different letters mean significant differences ($p < 0.05$) by one-way ANOVA followed by Tukey's test.

Abreu et al. (2016) also did not find significant differences related to the parameters of growth rate and doubling time, in the benthic diatom, *Navicula* sp., when comparing the Conway medium with a solid residue of the BFT effluent. Still corroborating with these authors, regarding to cell density, the culture medium also enhanced the effluent. This fact is probably related to the composition not only of nitrogen and phosphorus sources (macronutrients), but also of micronutrients, which are extremely important for the mitochondrial pathways of microalgae (Safi, Zebib, Merah, Pontalier, & Vaca-Garcia, 2014).

The precocity of the log phase in the condition containing only the BFT effluent (E100) could increase the amount of effluent treated (considering that microalgae cultures should always be inoculated in log phase) and biomass production. In view of this fact, we decided to analyze the data of this study again, simulating the end of the experiment on the fourth day of cultivation. The new verification was performed for the growth parameters MCD, K, and DT and is shown in Table 2. The conditions that contained the BFT effluent showed similar performances among each other, and above the E0 conditions, except for the MCD parameter, which was higher at the E100 condition, when compared to the other two.

Table 2. Mean values (± standard deviation) of maximum cell density (MCD), growth rate (K) and doubling time (DT) for *Chlorella vulgaris* analyzed from the 1st to the 4th day of culture.

Parameter	Treatment		
	E0	E50	E100
MCD (10^4 cells mL^{-1})	117.5 ± 17.5^a	280 ± 73.5^a	365 ± 45.69^b
K (div day^{-1})	1.18 ± 0.07^a	1.59 ± 0.14^b	1.73 ± 0.06^b
DT (days)	0.85 ± 0.05^a	0.63 ± 0.06^b	0.6 ± 0.02^b

Different letters mean significant differences ($p < 0.05$) by one-way ANOVA followed by Tukey's test.

Water quality and nutrient removal

The water quality parameters of the different growth regimes are shown in Table 3. The comparisons were made between the measurements (initial and final) of each condition and between all growth conditions. The water temperature did not show significant differences between the measurements, however, the E0 condition presented values significantly higher than the E50 and E100 conditions. The dissolved oxygen did not present significant differences between the growth regimens. There were differences between the measurements in the conditions that contained BFT effluent (E50 and E100). The pH had a significant difference between the growth regimens but were statistically equal between the initial and final measurements. The salinity presented significant similarity in the E0 condition however, it differed between the measurements in the E50 and E100 conditions. It was also verified a significant difference in salinity, when comparing the conditions with BFT effluent and E0.

Table 3. Mean values (± standard deviation) of water temperature (ºC), dissolved oxygen (DO; mg L^{-1}) and pH for different growth regime.

Parameter	Treatment					
	E0		E50		E100	
	Initial	Final	Initial	Final	Initial	Final
Temperature (ºC)	27.39 ± 0.8^{aA}	27.81 ± 0.5^{aA}	24.38 ± 0.3^{aB}	24.42 ± 0.2^{aB}	24.51 ± 0.4^{aB}	24.42 ± 0.1^{aB}
DO (mg L^{-1})	4.22 ± 0.3^{aA}	4.58 ± 0.2^{aA}	5.21 ± 0.7^{aA}	3.96 ± 0.1^{bA}	5.62 ± 0.2^{aA}	4.51 ± 0.5^{bA}
pH	7.95 ± 0.2^{aA}	7.62 ± 0.3^{aA}	6.15 ± 0.7^{aB}	6.45 ± 0.4^{aB}	6.52 ± 0.1^{aB}	6.35 ± 0.5^{aB}
Salinity	0.72 ± 0.1^{aA}	0.85 ± 0.1^{aA}	5.21 ± 0.0^{aB}	4.45 ± 0.1^{bB}	5.62 ± 0.1^{aB}	4.61 ± 0.2^{bB}

Different lowercase letters mean significant differences between the measurements (initial and final), while the different capital letters represent significant differences in the same line. ($p < 0.05$) by one-way ANOVA followed by Tukey's test.

Salinity cannot be a limiting factor in this study. Although this parameter was significantly different between treatments, all the conditions were acclimatized (by four days) and the literature affirms that the microalga *Chlorella vulgaris* can develop in salinities of 0 to 30 (Alyabyev et al., 2007; Yeesang & Cheirsilp, 2011). Regarding temperature, *C. vulgaris* is considered as a thermotolerant microalga (Trivedi et al., 2019) and in this study, the high temperature did not affect the growth of this species.

The CO_2 non-use was probably a positive factor for this cultivation, since the injection of such gas would cause culture acidification and could lead to inhibition of growth (Bautista-Chamizo, Borrero-Santiago, Manoela, DelValls, & Riba, 2018), after all, the pH of the culture did not increase at the end of the culture. This strategy was able to significantly reduce the costs of production of *Chlorella vulgaris*, since this gas accounts for around 30% of production costs (Chisti, 2007).

The data for quantitative removal of nitrogen compounds (nitrite, nitrate and ammonia) and phosphorus are given in Table 4. The nitrogen and phosphorus removal at the end of the E100 cultivation period was measured to assess the efficiency of *Chlorella vulgaris* nutrient removal from BFT effluent. About 80% of the nitrogenous sources of the E0 group were removed, and almost 50% of the phosphorus source (orthophosphate) was removed.

Table 4. Bioremediation efficiency of nitrogenous and phosphatic compounds by the microalga *Chlorella vulgaris* in the biofloc wastewater (E100) used in the Nile tilapia fingerlings farming.

	NH_4 (mg L^{-1})	NO_2 (mg L^{-1})	NO_3 (mg L^{-1})	PO_4 (mg L^{-1})
Initial	0.55 ± 0.06	1.23 ± 0.03	91.21 ± 0.03	7.66 ± 0.07
Final	0.23 ± 0.07	0.08 ± 0.02	5.84 ± 0.03	3.15 ± 0.09
% Remediation	79.3	80.1	93.6	48.3

The nutrients used for the formulation of the Provasoli medium provide a balanced diet of macronutrients and micronutrients, which are fundamental for the cellular activities of microalgae. As effluents, the presence and quantity are relative and some of the nutrients are available in different forms and may cause metabolic deficiencies. The capacity of the microalga in assimilating nitrogenous compounds was also perceptible and greater than that of the phosphate compounds, when the bioremediation efficiencies of the BFT effluent components were analyzed. However, it should be emphasized that even with all the care taken with wastewater disinfection, the nitrogen sources could have been also assimilated by nitrifying bacteria (Shan & Obbard, 2001).

Nutrient removal efficiency depends on the concentrations of nitrogen and phosphorous present in water (Goldberg & Cohen, 2006). In a study conducted by Li et al., 2011, the *C. vulgaris* removed 89.1% of total nitrogen and 80.9% of total phosphorus from a tertiary municipal wastewater, in 14 days of cultivation. Similar amounts of nitrogen compounds were also removed in this study.

The P is an important macronutrient for microalgae growth, and the microalgae assimilate phosphorus to produce phospholipids, ATP and nucleic acids (Ji et al., 2014). When using *C. vulgaris* for nutrient removal from urban wastewater, Sing, Birru and Sibi (2017) observed that *C. vulgaris* removed between 93.4 - 98.4% of total phosphorus, that are higher results than those found in this study. High phosphate concentrations may lead to unhealthy cyanobacteria blooms in culture systems (Anderson, Glibert, & Burkholder 2002; Oliveira et al., 2019), as well as the dissolved organic nitrogen (Anderson et al., 2008), which may cause problems to cultivated species (Kangur, Kangur, Kangur, & Laugaste, 2005).

Regarding the removal of the nitrogen and phosphorus compounds, the values of our study may seem considerably lower when compared to some of the values described in literature, as in some studies they were up to 100% for nitrogen and phosphorus (e.g. Martinez, Sanchez, Jimenez, El Youfi, & Munoz, 2000; Magnotti, Lopes, Derner, & Vinatea, 2016). Even so, it is important to emphasize that the cultures in these studies were initially stocked with different densities, which causes faster assimilations and larger amounts when inoculated in higher density. Depending on the purpose, the water after cultivation of *C. vulgaris* would require further treatment to remove nitrate and orthophosphate residual (WHO, 2004).

Unfavorable conditions, whether due to the absence or excess of some nutrient, may result in changes in morphology (Martínez, Ascaso, & Orús, 1991), primary composition (Liu, Wang, & Zhou, 2008; Ansilago, Ottonelli, & De Carvalho, 2016) and the production of pigments (Yamaguchi, 1996). The use of effluents from intensive aquaculture systems has potential as an alternative culture medium for microalgae (Trivedi et al., 2019). In the present study, the use of BFT effluent used in fish fingerlings farming also presented potential to produce microalga biomass.

Conclusion

In this sense, the use of BFT wastewater from a Nile tilapia fingerlings farming presented a satisfactory performance growth of the green microalga *C. vulgaris*. In addition to the biomass produced, there was elimination of costs regarding the reagents used in the culture medium formulation. Furthermore, the microalga assimilated 93.6% of nitrate and 48.3% of orthophosphate, which improves the water quality of this wastewater. However, possible toxic effects were not analyzed on the biomass produced on the effluent.

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